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Potassium glutamate as a transcriptional inhibitor during bacterial osmoregulation

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Potassium glutamate accumulates upon hyper-osmotic shock and serves as a temporary osmoprotectant. This salt leads to transcriptional activation of sets of genes that allow the cell to achieve long-term adaptation to high osmolarity. The current experiments show that potassium glutamate also acts as an inhibitor of bulk cellular transcription. It can do so independent of the involvement of macromolecular repressors or activators by virtue of its ability to directly inhibit RNA polymerase binding to ribosomal promoters. Thus, potassium glutamate mediates a global transcription switch by acting differentially on RNA polymerase at sets of genomic promoters that differ in their built-in direct response to this salt.

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Introduction

Escherichia coli must adapt to increases in osmotic pressure to survive its passage along the human GI tract (Foster and Spector, 1995). The consequence of lack of osmotic adaptation is the loss of water and turgor pressure needed for metabolism and cell division (Wood, 1999). Survival depends on the accumulation of small molecule osmoprotectants that prevent loss of water and are also compatible with normal biochemical events. Among the osmolytes in the lower GI tract are short-chain fatty acids, predominantly acetate (Cummings *et al*, 1987), that additionally challenge cell survival by accumulating in the cytoplasm, transiently lowering pH and altering cellular metabolism (Roe *et al*, 1998, 2002).

When growing *E. coli* are subject to osmotic shock, wholesale changes in growth, metabolism and gene expression occur (Wood, 1999). Many of these are common to all forms of hyperosmotic shock and some are unique changes caused by specific external osmolytes. Hyperosmotic shock stops cell growth immediately and growth does not resume until a series of adaptive biochemical events unfold (Jovanovich *et al*, 1988). The length of the growth delay depends on the degree of the shock, the mix of osmolytes present and the availability of external osmoprotectants (Jovanovich *et al*, 1988).

As part of this adaptive strategy, cells typically quickly accumulate high concentrations of potassium glutamate (Dinnbier *et al*, 1988; Cayley *et al*, 1991), which provides temporary protection. The potassium salts of glutamate and to a lesser extent acetate are thought to be the primary intracellular ions (Cayley *et al*, 1991), and at normal concentrations potassium glutamate typically stabilizes DNA–protein interactions compared to the equivalent chloride salt (Leirmo *et al*, 1987). Nonetheless, higher concentrations have the capacity to disturb cellular metabolism, making potassium glutamate a less preferred osmolyte for long-term protection against hyper-osmotic shock. Accumulation is accompanied by a gene expression program that leads to the transport of more effective osmoprotectants if available as well as *de novo* synthesis of other osmoprotectants (Jovanovich *et al*, 1988; Kempf and Bremer, 1998). These are generally neutral compounds that can accumulate to very high levels without inducing severe disturbances in cellular metabolism. Their accumulation allows growth to resume efficiently in the presence of high external osmotic pressure. The high internal concentration of potassium glutamate is apparently not retained under conditions of restored growth in hyper-osmotic conditions (Dinnbier *et al*, 1988).

The changes associated with the intracellular accumulation of high concentrations of potassium glutamate or acetate include a re-programming of the cellular transcription apparatus (Kirkpatrick *et al*, 2001; Oh *et al*, 2002; Weber and Jung, 2002; Cheung *et al*, 2003; Polen *et al*, 2003). Most prominent among the induced transcripts are those made by the sigma38 (rpoS) form of RNA polymerase. A high concentration of potassium glutamate has been proposed to act directly on certain sigma38 promoters to activate them *in vivo*, without the need for the action of macromolecular repressors or activators (Lee and Gralla, 2004).

Array analyses (see above references) have identified individual genes activated by high salt and acetate, but little is known about how potassium glutamate or acetate accumulation affects bulk transcription during the adaptation phase when growth is halted. Immediate repression of bulk transcription would be advantageous to the cell as an accompaniment to the temporary shut-down of growth. Total cellular transcription, as measured by P-32 incorporation into pulse-labeled RNA, was reported to be reduced by half 15 min after a 300 mM NaCl shock (Afflerbach *et al*, 1998). This time is in the middle of the adaptation period when the gene expression program is already underway and well after growth has halted (Jovanovich *et al*, 1988). This reduction in newly synthesized RNA is thought to be due to repression of ribosomal transcription because under moderate to rapid growth conditions the large majority of new cellular transcripts are ribosomal (Bremer and Dennis, 1996).

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Neither the molecular basis nor the timing of this repression of bulk transcription after the challenge of cells with salt or acetate is known. Although the ribosomal RNA promoters account for the bulk of cellular transcription during moderate to rapid growth (Bremer and Dennis, 1996), there have not been measurements of the effects of osmolytes on transcription from these promoters *in vivo*. It is not known whether the repressive effects of osmolytes are direct or are instead mediated by macromolecular regulators.

The current study is aimed at understanding the effect of osmotic shock on repression of bulk cellular transcription. One goal is to learn how the ribosomal promoters respond to hyperosmolarity just after the initial shock and during the adaptation that allows resumed growth. Another is to clarify the molecular basis of these events. The data will lead to a model in which repression of bulk transcription is proposed to be a consequence of the direct action of various osmolytes on sigma70 RNA polymerase binding to the ribosomal promoters. Remarkably, the same small molecule, potassium glutamate, can either repress or stimulate transcription depending on the transcription complex and this differential effect is proposed to be a critical aspect of the osmotic response pathway.

Results

During rapid growth most cellular transcription derives from initiation at the seven nearly identical ribosomal promoter regions. These regions are coregulated and expression from the major P1 promoters accounts for the bulk of ribosomal transcription (Paul *et al*, 2004). Prior work has established a primer extension assay for new ribosomal transcription, and we used this to follow changes upon osmotic shock (Zhi *et al*, 2003). The primer hybridizes within the 5′ leader region of the unprocessed preribosomal RNA of four of the seven promoters. Because mature ribosomal RNA does not contain this leader, this assay is taken as a measure of newly initiated transcripts.

The physiological context for these RNA measurements is shown in Figure 1. Cells are grown to early to mid-log phase and then challenged by addition of 0.4 M NaCl (addition noted by an arrow in the figure). The rise in optical density halts immediately (compare to unchallenged growth curve), reflecting the known inhibition of growth that is triggered by hyperosmotic shock (Jovanovich *et al*, 1988). After a lag of 20 min growth apparently resumes, signaling the completion of the adaptation period. RNA was isolated moderately early in the adaptation period (5 min postshock; broken arrow in Figure 1) and was probed to reveal the preribosomal RNA transcripts (Figure 1, inset). Compared to a control sample lacking added salt, the signal is very weak (lane 2 versus the lane 1 control). We infer that hyperosmotic shock severely inhibits ribosomal transcription *in vivo*.

A similar experiment was performed using potassium acetate shock and is shown in Figure 2. The growth behavior (Figure 2) is similar to that seen with salt shock; there is an immediate halt, followed by a lag, in turn followed by resumed growth. In this case, the lag is much longer likely due to the toxic effects of imported acetate (Roe *et al*, 1998). Preribosomal RNA analysis 5 min after addition (broken arrow) shows that potassium acetate also leads to a severe inhibition of ribosomal transcription (Figure 2, inset lane 2 compared to the lane 1 control).

Next, the kinetics of changed ribosomal transcription was monitored. This is important for two reasons. Most important, the initial event after hyper-smotic shock is the rapid import of potassium accompanied by the accumulation of glutamate (see Introduction). Other osmoprotectants accumulate more slowly than potassium glutamate, and it is this slower accumulation that likely completes the adaptation. Thus, very short-time measurements reflect primarily a potassium glutamate environment. In addition, ribosomal transcription can be affected by changes in the levels of chromosomal proteins Fis and H-NS (reviewed in Paul *et al*, 2004; Gralla, 2005). Such changes are also slow and if they occur are expected later in the adaptation period. Therefore, strong inhibition at the very earliest times should reflect increases in potassium glutamate levels rather than these other potential macromolecular effectors.

Figure 3A shows the time course of expression beginning immediately after the salt shock and extending throughout the adaptation period. These data reflect primarily newly initiated ribosomal transcripts, and thus are an approximate measure of transcription initiation (see above). There are two important aspects to these data. First, newly synthesized RNA is at much reduced levels immediately after addition of salt (lane 1 and also compare lanes 1 and 2 of Figure 3B). This implies that the inhibition is a primary response and does not require changes in levels of the neutral osmoprotectants that accumulate more slowly. Potassium glutamate

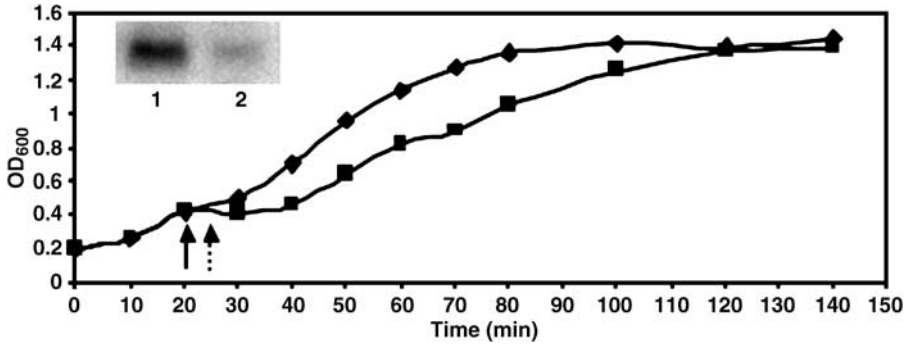

Figure 1 Effect of salt on growth and ribosomal RNA transcription. K12 cells grown in LB media and at OD = 0.4 were split and half received NaCl to a final concentration of 400 mM (squares) and half-received water (diamonds). The time of addition is marked by a solid arrow. After 5 min (broken arrow) RNA was isolated from cells receiving water (lane 1) and salt (lane 2), and analyzed by primer extension. The experiment was repeated three or more times with similar results.

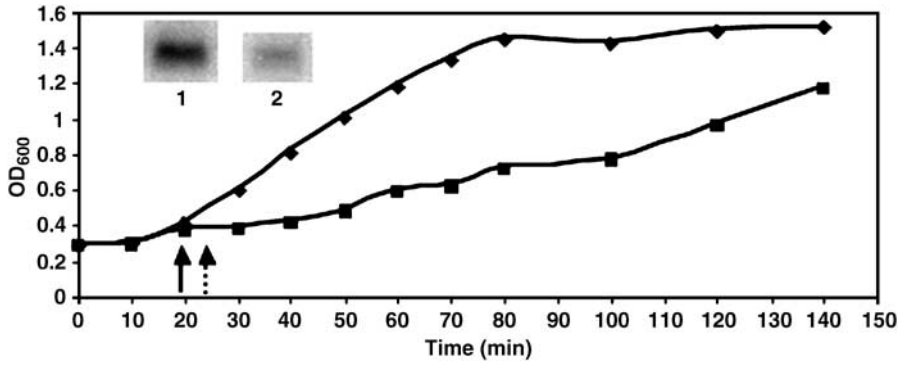

Figure 2 Effect of potassium acetate on growth and ribosomal RNA transcription. K12 cells grown in LB media and at OD = 0.4 were split and half received potassium acetate (squares) to a final concentration of 100 mM and half-received water (diamonds). The time of addition is marked by a solid arrow. After 5 min (broken arrow) RNA was isolated from cells receiving water (lane 1) and acetate (lane 2) and analyzed by primer extension. The experiment was repeated three or more times with similar results.

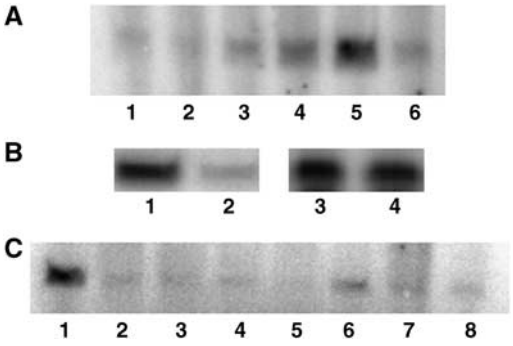

Figure 3 Time course of salt-induced changes in ribosomal RNA transcription. Cells were stressed with NaCl as in Figure 1 and ribosomal RNA was analyzed by primer extension at various times thereafter. The experiment was repeated three or more times with similar results. (**A**) Rich media (LB): immediately after salt induction (lane 1) and 5, 10, 20, 40 and 60 min after induction (lanes 2–6). (**B**) Cells were transferred either to water (lanes 1 and 3) or salt (lanes 2 and 4), and immediately harvested and then probed for r-RNA (lanes 1 and 2) or gadB RNA (lanes 3 and 4). (**C**) Minimal media (M9 + glucose): just after induction with salt (lane 2) and 10, 20, 40, 60, 80 and 120 min later (lanes 3–8). Lane 1 is without induction. The cell doubling time was 1 h and the maximum growth delay caused by salt was 30 min.

has been reported to accumulate in this same short time (Dinnbier *et al*, 1988). The specificity of the inhibition is confirmed by analysis of the effect on gadB RNA, which is shown to be largely unaffected by the salt shock (Figure 3B, lane 3 versus lane 4).

Second, the data show that after this initial strong inhibition there is a slow recovery of ribosomal transcription (Figure 3A, lanes 2–5). The kinetics of this recovery follows that of the apparent resumption of growth and roughly correspond to decreases in intracellular potassium glutamate levels (Dinnbier *et al*, 1988). As the cells approach the slow stationary phase of growth, the amount of r-RNA is reduced (lane 6) as already known to be the case (Paul *et al*, 2004). We infer that ribosomal transcription is immediately halted upon hyper-osmotic shock and only recovers as the intracellular environment adapts to allow renewed growth under high salt conditions.

The complex LB media used in these experiments may contain neutral osmoprotectants such as glycine betaine or related molecules. In minimal media, the accumulation of neutral osmoprotectants relies on known synthetic pathways and cells primarily produce the osmoprotectant trehalose (Kempf and Bremer, 1998). New transcription in minimal media begins after a lag of several minutes, and is maintained during the adaptation period (Jovanovich *et al*, 1988). Figure 3C shows that salt shock in minimal media leads to an immediate and strong inhibition of ribosomal transcription (compare lanes 1 and 2). The level of inhibited transcription appears to be similarly low to that in LB; the level prior to shock is lower than in LB, as is expected from the lower levels known to accompany the reduced doubling time in minimal media. There is a very modest restoration of transcription during the adaptation period (lanes 2–8), but this never reaches high levels, as expected for slow growth in minimal media (Bremer and Dennis, 1996). Overall, the pattern is similar in rich and minimal media, with an immediate inhibition of ribosomal transcription followed by a slow recovery.

Transcription of ribosomal RNA is known to be strongly affected by the nucleotide ppGpp and the macromolecules Fis and H-NS (Paul *et al*, 2004; Gralla, 2005). There have been no reports of rapid changes in Fis or H-NS activities, but kinetic studies after growth downshifts suggest that ppGpp levels might change over the time scale of these experiments (Murray *et al*, 2003). To assess the potential contribution of changing ppGpp levels to the observed rapid reduction in transcription, a quantitative experiment was performed to compare the salt-induced reduction in r-RNA transcription of wild-type cells with those deficient in ppGpp production.

The results, done in LB media, are shown in Figure 4. Cells with low ppGpp levels are seen to produce the same amount of ribosomal RNA as wild-type cells (lanes 1 versus 7), and this amount is lowered five-fold for both wild type and mutant cells immediately after shock (lanes 2 versus 8) and 5 min later (lanes 3 versus 9). A parallel comparison was performed with cells deficient in the transcriptional activator Fis. In this case, the initial RNA level is lower (lanes 1 versus 4), as expected. Both strains strongly reduce RNA levels after salt shock (lanes 2 and 5) although the Fis-deficient strain appears to show some difficulty in recovery (lanes 3 versus 6), again as may be expected. Basically, the data show that there is no detectable contribution made by ppGpp or Fis to the rapid inhibition of r-RNA expression upon salt challenge. The available data do suggest that macromolecular regulators

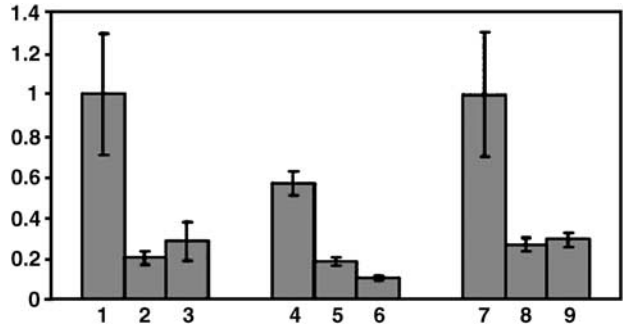

Figure 4 Comparison with ppGpp and Fis-deficient strains. Cells from the three strains were stressed with NaCl as in Figure 1 and ribosomal RNA was analyzed by primer extension either before stress (lanes 1, 4 and 7), immediately after (lanes 2, 5 and 8) or 5 min later (lanes 3, 6 and 9). Lanes 1–3 are wild type, lanes 4–6 are Fis-deficient and lanes 7–9 are ppGpp-deficient. The data was from a single set of experiments carried out in quadruplicate and averaged.

might contribute to total amounts of r-RNA at later times during the recovery period (see Afflerbach *et al*, 1998 for H-NS). However, the initial strong inhibition appears to be mostly independent of the known regulators of r-RNA transcription.

It is also possible that changes in the leader r-RNA turnover rate could contribute to the rapid reduction in level observed. This process, measured previously in a plasmid expression context, was estimated to have a halftime of slightly more than a minute (Schaferkordt and Wagner, 2001). We conducted rifampicin challenge experiments using the current experimental protocol and found a similar r-RNA leader half-life after salt challenge (data not shown), suggesting that enhanced turnover is not the cause of the observed reduction in transcription.

This immediate severe reduction in ribosomal transcription is roughly coincident with a rapid increase in intracellular potassium glutamate levels. As discussed above, array analyses have shown that a number of promoters, primarily those transcribed by sigma38 RNA polymerase, are activated under these conditions. Sigma70 promoters show diverse responses to potassium glutamate, and both stimulation and repression have been noted *in vitro* (Leirmo *et al*, 1987; Lee and Gralla, 2004). Ribosomal promoters (sigma70-controlled) are known to be unusually sensitive to salt inhibition (Ohlsen and Gralla, 1992). To assess how ribosomal transcription responds to increases in potassium glutamate levels, *in vitro* transcription was carried out.

Figure 5A shows the transcription response of the supercoiled rrnb promoter as potassium glutamate levels are increased *in vitro*. The data show that high concentrations of potassium glutamate lead to strong reductions in ribosomal transcription (see also Zhi *et al*, 2003). The data suggest that the increasing potassium glutamate concentration known to occur upon osmotic shock is expected to strongly inhibit ribosomal transcription.

The addition of potassium acetate also inhibits ribosomal transcription *in vivo* (see Figure 2) and *in vivo* acetate anion is known to become rapidly concentrated in the cytoplasm (Roe *et al*, 1998). This raises the possibility that the reduction in transcription is caused directly by acetate salts acting at the ribosomal promoters. To assess this possibility, the *in vitro*

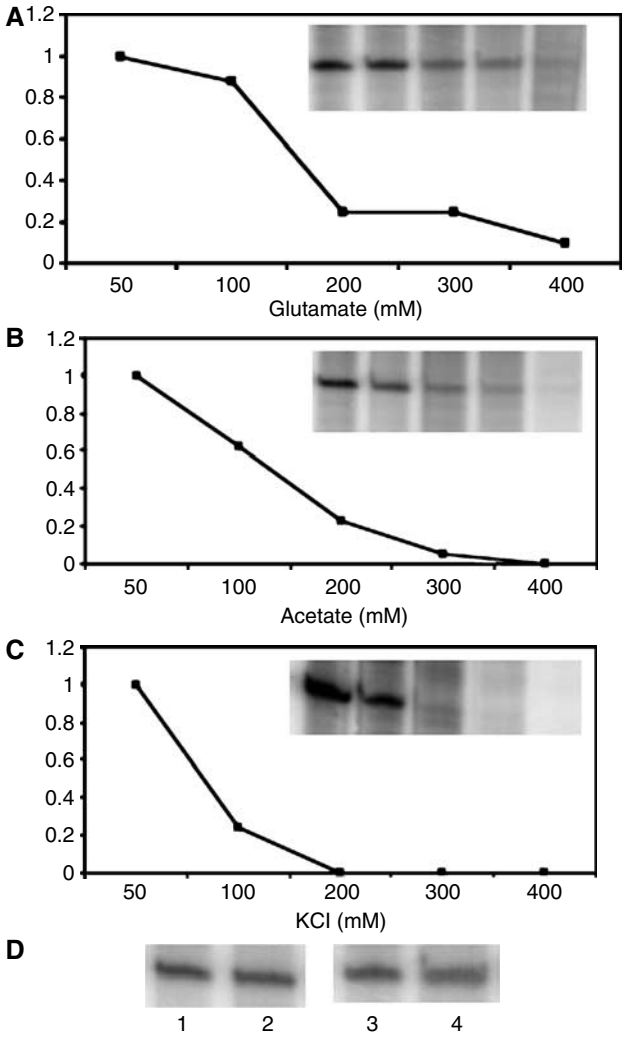

Figure 5 Effect of salts on rrnb transcription *in vitro*. Single round transcription was assayed in the presence of the indicated concentrations of potassium glutamate (**A**), potassium acetate (**B**) and potassium chloride (**C**). Insets show transcripts from the lowest to the highest of the five concentrations tested. (**D**) lac UV5 DNA was transcribed at 50 mM KCl (lane 1) or 200 mM KCl (lane 2) or 50 mM potassium glutamate (lane 3) or 200 mM potassium glutamate (lane 4). The data are from the average of two to four experiments.

experiments were repeated as a potassium acetate titration and the results showed strong inhibition (Figure 5B). These inhibitory effects of salts of acetate and glutamate can be viewed as part of the known sodium chloride-sensitivity of ribosomal transcription (Ohlsen and Gralla, 1992), reproduced here for potassium chloride (Figure 5C). As a comparison, we show that the common lac UV5 test promoter is only slightly inhibited at 200 mM salts compared to 50 mM (Figure 5D: KCl, compare lanes 1 and 2; potassium glutamate, compare lanes 3 and 4), the same range that leads to severe reductions in ribosomal transcription (Figure 5A and C). Overall, we infer that the inhibition of ribosomal transcription upon accumulation of salts of glutamate or acetate *in vivo* can be explained in significant part by direct inhibition by salts at the salt-sensitive promoters.

Inspection of the salt inhibition data shows that although glutamate and chloride anions are both inhibitory, transcription is higher with glutamate, consistent with the known

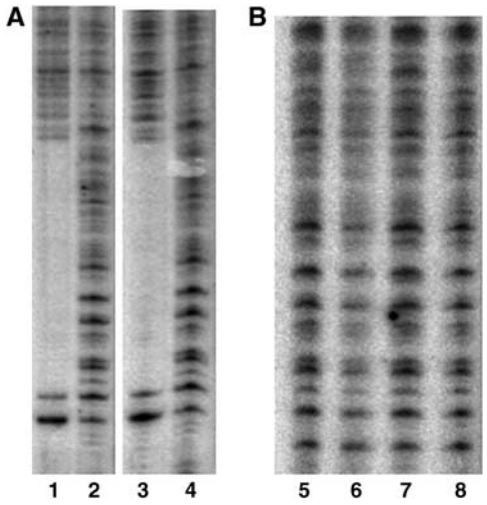

Figure 6 DNase footprints at low and high potassium glutamate concentrations. The procedure followed that of Ohlsen and Gralla (1992) done on the same promoter and corresponds to the full open complexes formed in the absence of competitor. (**A**) 50 mM potassium glutamate: lane 1: supercoiled DNA with RNA polymerase; lane 2: supercoiled DNA with BSA; lane 3: linear DNA with RNA polymerase; lane 4: linear DNA with BSA. (**B**) 300 mM potassium glutamate: lane 5: supercoiled DNA with BSA; lane 6: supercoiled DNA with RNA polymerase; lane 7: linear DNA with BSA; lane 8: linear DNA with RNA polymerase.

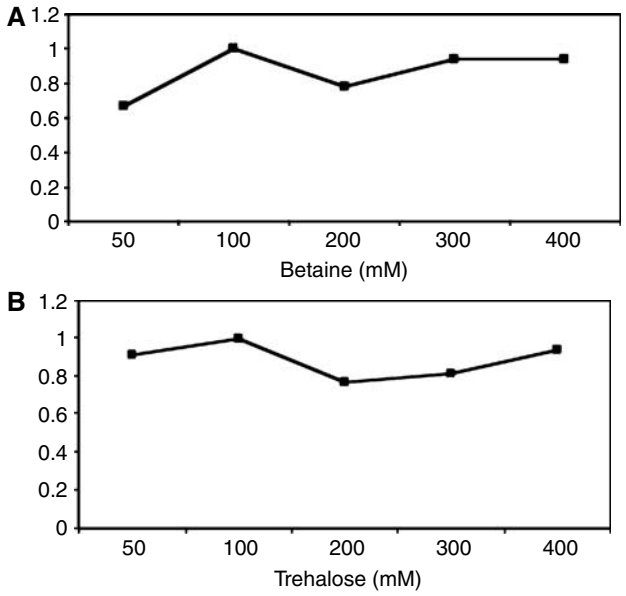

Figure 7 Effect of neutral osmoprotectants on rrnb transcription *in vitro*. Single round transcription was assayed in the presence of the indicated concentrations of glycine betaine (**A**) and trehalose (**B**). The data are from duplicate experiments.

ability of general transcription to be increased when glutamate is substituted for chloride (compare fraction of transcription remaining at 300 mM) (Leirmo *et al*, 1987). Apparently, the built-in salt sensitivity of the rrn promoters is sufficiently severe so as to direct strong inhibition when potassium glutamate accumulates, allowing physiologically appropriate inhibition of ribosomal transcription upon hyper-osmotic shock.

The nature of the sensitivity to potassium glutamate is not known. When potassium glutamate accumulates upon osmotic shock, it induces certain sigma38 promoters and at osmY this is proposed to occur by triggering escape of RNA polymerase already bound and poised at that promoter (Lee and Gralla, 2004). To test whether potassium glutamate is blocking binding or escape of RNA polymerase at the rrnb promoter DNase footprints were carried out, as described previously at the same promoter without potassium glutamate (Ohlsen and Gralla, 1992). The data compare promoter occupancy at 50 mM potassium glutamate where transcription is high to 300 mM where transcription is low. At 50 mM potassium glutamate, where transcription is high (Figure 5A), a clear open complex footprint is observed (Figure 6A, lanes 1 versus 2). At 300 mM potassium glutamate, where transcription is very low, a footprint is either absent or barely detectable (Figure 6B, lanes 5 versus 6). These footprints were carried out on supercoiled DNA, using primer extension methods (Gralla, 1985), to allow direct comparisons with the transcription experiments. Because supercoiling appears to change upon osmotic shock (Higgins *et al*, 1988; Hsieh *et al*, 1991) and because ribosomal promoters are sensitive to changes in supercoiling (Ohlsen and Gralla, 1992), the footprints were repeated on linearized DNA. The data show again that high potassium glutamate concentrations eliminate RNA polymerase binding (similar signals in lanes 7 and 8 compared to the footprint seen by comparing lanes 3 and 4). We infer that the high concentrations of potassium glutamate that accumulate upon osmotic shock would be expected to block the binding of RNA polymerase to the ribosomal promoters.

The kinetic data (above) show that ribosomal transcription eventually recovers as cell growth resumes. Renewed growth requires the accumulation of neutral osmoprotectants, most likely glycine betaine in rich media and trehalose in minimal media (Kempf and Bremer, 1998). Figure 7A and B shows that high concentrations of these components do not inhibit rrnb transcription. These experiments were conducted over the same range of concentrations over which salts were strongly inhibitory (Figure 5). We suggest that the accumulation of these neutral osmoprotectants contributes to the restoration of ribosomal transcription needed for resumption of growth (Figures 1 and 2).

Discussion

These experiments define a pathway that cells use to control bulk transcription during hyper-osmotic shock. The primary attribute of the proposed pathway is that it centrally involves the direct inhibitory effect of potassium glutamate on RNA polymerase at the ribosomal promoters. The effect is promoter-selective as the ribosomal promoters are especially sensitive to inhibition by salts compared to other promoters. This transcription inhibition is accompanied by an opposite effect of potassium glutamate, namely the stimulation of a large number of genes, many transcribed by sigma38 (Weber and Jung, 2002; Cheung *et al*, 2003) and at least some of which appear to be directly stimulated by the salt (Lee and Gralla, 2004). Thus, a high level of potassium glutamate triggers a global transcription switch, in significant part because some promoters are designed to transcribe poorly and others to transcribe better under these conditions. Other important regulatory events include secondary effects of this potassium

glutamate regulation, for example the shutdown of ribosomal protein production (Weber and Jung, 2002), which inevitably occurs when ribosomal RNA levels are lowered (Nomura, 1999).

Potassium is imported immediately upon loss of turgor pressure, and this is quickly followed by glutamate production; this is the first line of defense against hyperosmotic shock (see Introduction). The above data show that ribosomal transcription, which accounts for the bulk of cellular transcription at moderate to high growth rates (Bremer and Dennis, 1996), is inhibited in the same short time. At slightly longer times, a different and stimulatory transcription program begins, also assisted by potassium glutamate accumulation, and this contributes to the accumulation of superior neutral osmprotectants. The current data show that ribosomal transcription slowly reactivates during this adaptation and recovery phase. Both the inhibition of ribosomal transcription by potassium glutamate (Figures 3 and 4), its restoration by neutral osmoprotectants (Figures 3 and 7) and the stimulation of a sigma38 promoter (Lee and Gralla, 2004) can be reproduced *in vitro* in the absence of macromolecular activators or repressors. These data support the proposal that osmotic regulation has a very large component of the direct effects of potassium glutamate and neutral osmoprotectants on RNA polymerase at diverse promoters.

The data show that the growth inhibition caused by 100 mM potassium acetate is severe and that it is also accompanied by strong inhibition of ribosomal RNA synthesis. Acetate and related short-chain fatty acids are abundant fermentation products in the human large intestine and can be present at concentrations above 100 mM (Cummings *et al*, 1987). Acetate anion is known to accumulate in the bacterial cytoplasm and to temporarily disturb metabolism (Roe *et al*, 1998; Roe *et al*, 2002). The current data suggest that the unusual salt-sensitivity of the ribosomal promoters allows cellular resources to be conserved while the cell adapts to these perturbations by ensuring that bulk transcription is inhibited during this time.

Ribosomal transcription is known to be regulated by chromosomal proteins and nucleotides (Paul *et al*, 2004) and r-RNA levels reflect changing ratios of effectors (Gralla, 2005). Inhibition occurs when conditions favor H-NS over Fis and also when changes favor ppGpp over ATP. Do changes in these four regulators contribute to the response to hyper-osmotic shock?

Nucleotide regulation is the more likely contributor as levels can change moderately rapidly upon downshift (see Murray *et al*, 2003 for ppGpp) and could in principle contribute to the rapid reduction in r-RNA levels observed here. ATP levels are known to increase immediately after hyper-osmotic shock (Ohwada and Sagisaka, 1987; Hsieh *et al*, 1991), and this would tend to increase ribosomal transcription, contrary to the immediate decrease that is observed here. We measured the effect of drastically lowering ppGpp levels on r-RNA transcription and none was evident (Figure 4), implying that this nucleotide makes little contribution to the observed rapid inhibition of r-RNA expression. DNA supercoiling has also been reported to increase upon osmotic shock (Higgins *et al*, 1988; Hsieh *et al*, 1991), but the existing data (Ohlsen and Gralla, 1992) show modest stimulation by supercoiling (also see Figure 5), making it unlikely that this contributes to the inhibition observed here. Overall, the net effects of these changes in the levels of effectors would be to increase rather than decrease transcription, implying that the inhibition is due to other factors.

With regard to regulation by macromolecules, a changing ratio of Fis to H-NS is less likely to make a major contribution to the observed immediate shutdown of ribosomal transcription as there are no reports of rapidly changing levels or activities of these proteins. The data of Figure 4 show that when this H-NS to Fis ratio is greatly increased by use of an Fis-deficient strain, there is no detectable effect on the rapid inhibition of ribosomal transcription. The data do hint at some deficiency in the later recovery of r-RNA levels. H-NS mutants have been reported to behave oppositely, yielding continued high levels of new r-RNA at 15 min postosmotic shock, as measured indirectly by incorporation of added P-32 into total soluble nucleic acid (Afflerbach *et al*, 1998). Collectively, these data suggest that the Fis to H-NS ratio makes little contribution to the rapid reduction in r-RNA transcription immediately upon salt shock, but may contribute during the subsequent recovery phase.

It is remarkable that the salt potassium glutamate can trigger a global transcription switch by acting differentially on cellular promoters without the participation of known macromolecule and small molecule regulators. The genome is apparently designed to contain promoters, whose DNA sequences direct dramatically different responses to the potassium glutamate that accumulates upon hyperosmotic shock. Some of these differences are related to the use of different sigma factors, but even for each sigma there are critical differences in how promoters respond. These built-in differences allow the cell to appropriately inhibit bulk transcription and also to trigger transcription of species that allow eventual resumption of growth under the new conditions. It will require further investigation to learn how promoter DNA sequences encode the information to direct either repression or activation of transcription in response to potassium glutamate accumulation, and our current focus is on testing whether the salt can induce conformational changes in transcription complexes that have different effects at different promoters (see Rosenthal *et al*, 2006).

Materials and methods

Cell growth and challenge

E. coli K12 was grown in LB medium (no added sugars) at 37°C until the OD_{600} reached 0.3–0.4. Cells were split and added to prewarmed concentrated NaCl, or potassium acetate (neutralized) or water, to the indicated final concentrations. At the indicated times, 1 ml samples were processed for RNA analysis (below). When indicated, M9-glucose media was used. The ppGpp-deficient relA strain RJ1847, and the Fis-deficient strain RJ1800, were kind gifts of Reid Johnson, UCLA.

In vivo r-RNA analysis

RNA was isolated from quickly centrifuged and frozen cells using the RNeasy Mini Kit (Qiagen). Primer extension used reverse transcriptase and the manufacturer's protocol (Promega). In this procedure, 1 μg RNA was mixed with 10 ng of end-labeled NSL166 primer (Zhi *et al*, 2003) (5′-GGGTGTGCATAATACGCC-3′), heated for 2 min at 80°C and cooled on ice. Primer extension was at 42°C for 1 h. The reaction was then cooled on ice, heated to 80°C in urea for 1 min and analyzed by 6% Urea–PAGE (in TBE). Radioactive bands were visualized and quantified by phosphorimager analysis. For GadB m-RNA analysis, the samples were probed with the primer described in Rosenthal *et al* (2006). To measure leader r-RNA turnover, rifampicin chase experiments were carried out.

Rifampicin was added to 50 μg/ml and cells were harvested, recovered and frozen within a minute and at subsequent times. Leader r-RNA was then assayed and compared to samples taken prior to rifampicin addition.

In vitro transcription (Lew and Gralla, 2004)
A measure of 1 μl of 25 nM supercoiled DNA (a derivative of ptH8, see Lew and Gralla, 2004) was mixed with 0.5 μl of 1 μM sigma 70 holoenzyme on ice. Other components were added in this order: 5 × buffer B (250 mM Tris–HCl pH 7.9, 15 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 500 μg/ml BSA), five times the final indicated concentrations of KCl, potassium glutamate or potassium acetate (both neutralized) and water to make 10 μl. For experiments with glycine betaine and trehalose 100 mM KCl was present, as transcription proceeds poorly in the absence of salts (Lee and Gralla, 2004). After a 37°C incubation for 15 min, 0.5 μl of NTP mix was added (8 μl 10 mM cold ATP, UTP, and GTP, 0.5 μl radioactive CTP, 8 μl of 5 mg/ml heparin, 4 μl water). The reactions were incubated for 5 min at 37°C. Transcripts, which stop at the inserted terminator, were analyzed by 6% Urea–PAGE and phosphorimager analysis, as above.

Footprinting (Ohlsen and Gralla, 1992)
Transcription complexes were assembled as described above, using supercoiled DNA (Gralla, 1985) unless otherwise stated. To maximize stability, 0.5 μl of NTP mix (8 μl 10 mM cold ATP and CTP plus 12 μl water) was added (slightly weaker protection was observed without added NTPs). After 5 min at 37°C, DNase I was added (20 ng for 50 mM potassium glutamate, 100 ng for 300 mM potassium glutamate) for 30 s at 37°C. The higher 100 ng of DNase 1 was required to compensate for inhibition by 300 mM potassium glutamate so as to provide a suitably equivalent digestion patterns. DNA was purified using the Qiagen PCR Purification Kit in the stated PB buffer. Footprints were revealed using Taq polymerase to extend the labeled primer (GGCAGTTTT AGGCTGATTTGG-3′) and analyzed by 6% Urea–PAGE and phosphorimager analysis, as above.

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